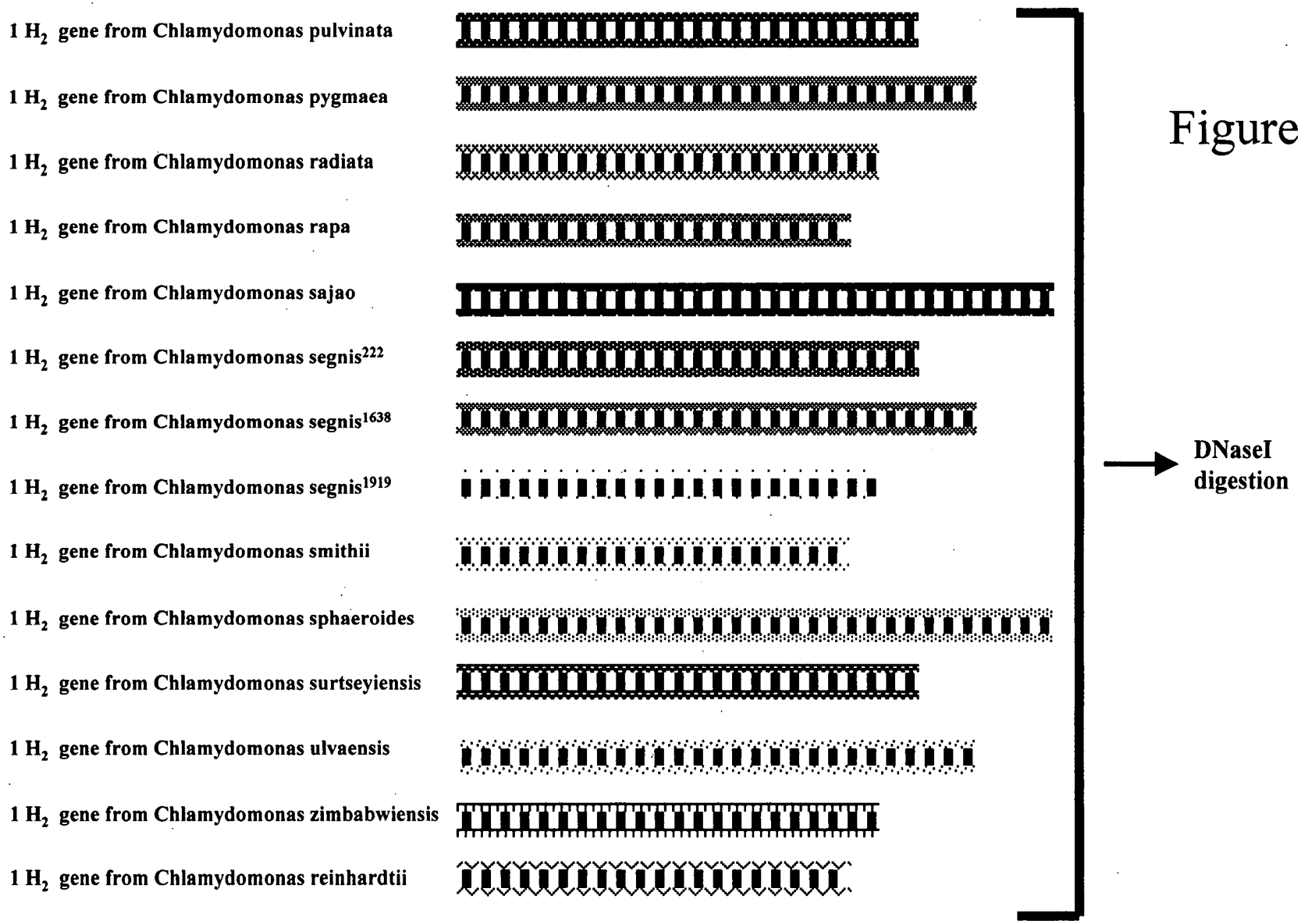
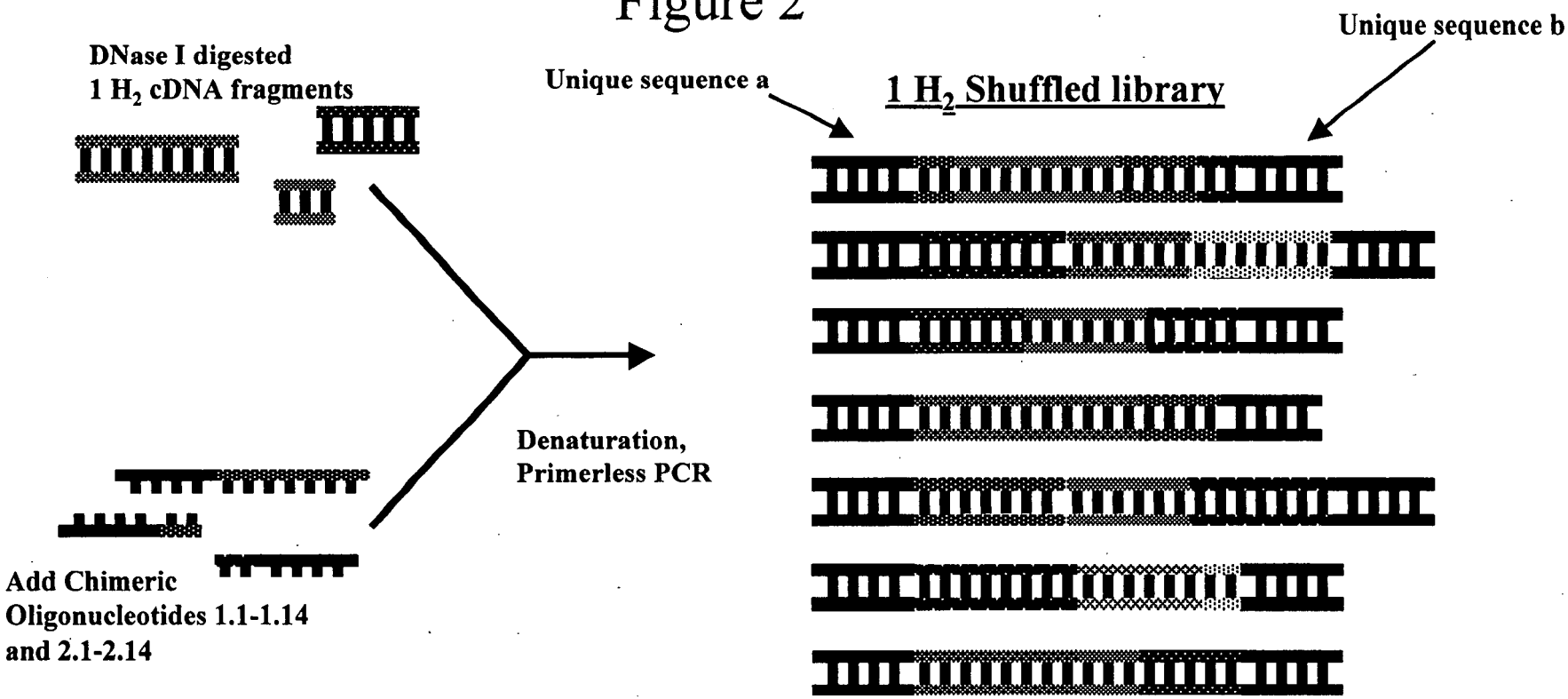

1 H_2 gene from Chlamydomonas pulvinata
1 H_2 gene from Chlamydomonas pygmaea
1 H_2 gene from Chlamydomonas radiata
1 H_2 gene from Chlamydomonas rapa
1 H_2 gene from Chlamydomonas sajao
1 H_2 gene from Chlamydomonas segnis^{222}
1 H_2 gene from Chlamydomonas segnis^{1638}
1 H_2 gene from Chlamydomonas segnis^{1919}
1 H_2 gene from Chlamydomonas smithii
1 H_2 gene from Chlamydomonas sphaeroides
1 H_2 gene from Chlamydomonas surtseyiensis
1 H_2 gene from Chlamydomonas ulvaensis
1 H_2 gene from Chlamydomonas zimbabwiensis
1 H_2 gene from Chlamydomonas reinhardtii
DNaseI digestion

Figure 1

Figure 2



Shuffled $2H_2$, $3H_2$, $4H_2$, and $5H_2$ libraries are created through the same method using chimeric oligonucleotides 3.1-3.14/4.1-4.14, 5.1-5.14/6.1-6.14, 7.1-7.14/8.1-8.14 and 9.1-9.14/10.1-10.14, respectively. Sequences 2 H_2, 3 H_2, 4 H_2, and 5 H_2 are flanked by unique sequences c and d, e and f, g and h, and i and j, respectively.

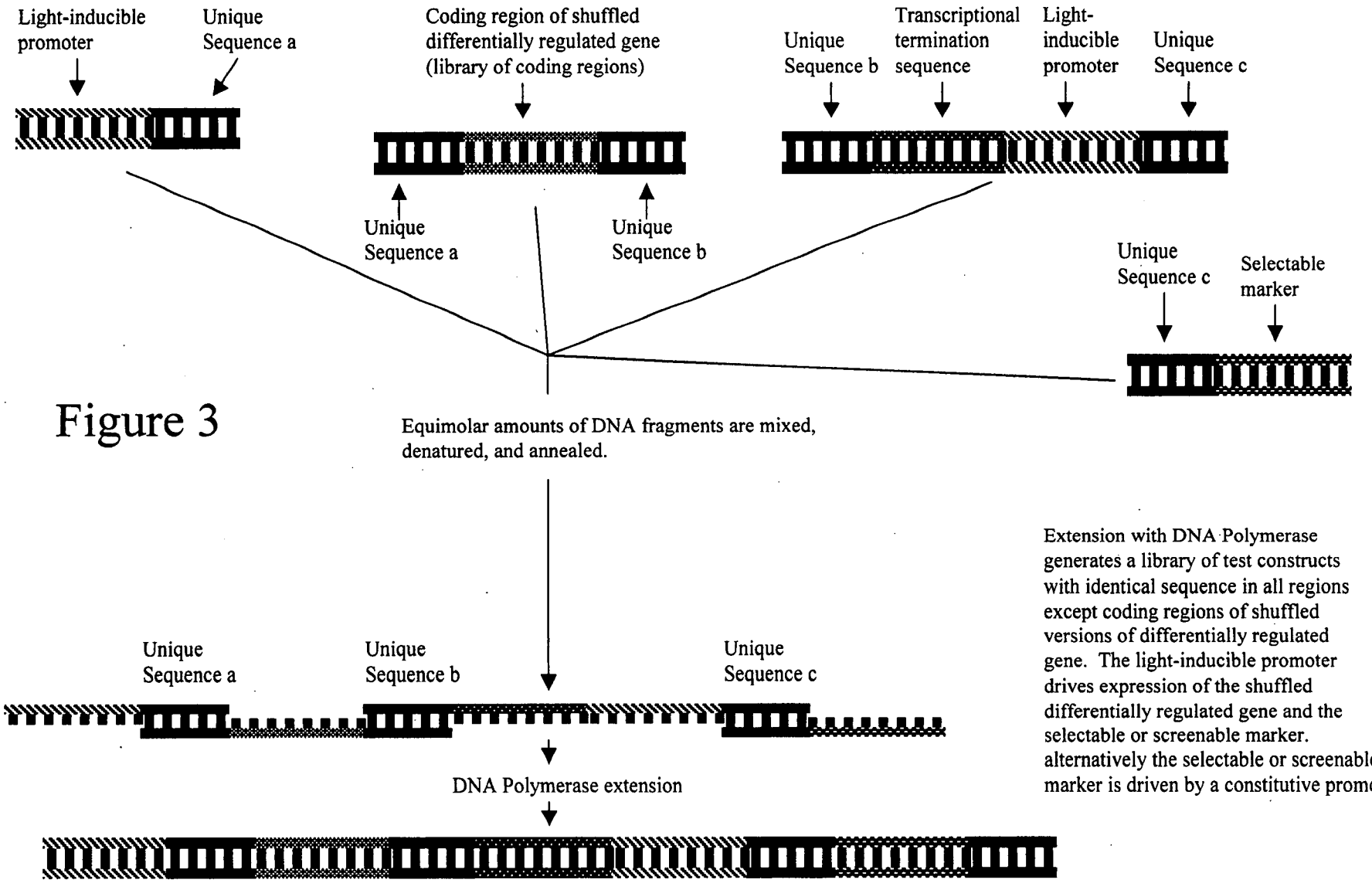
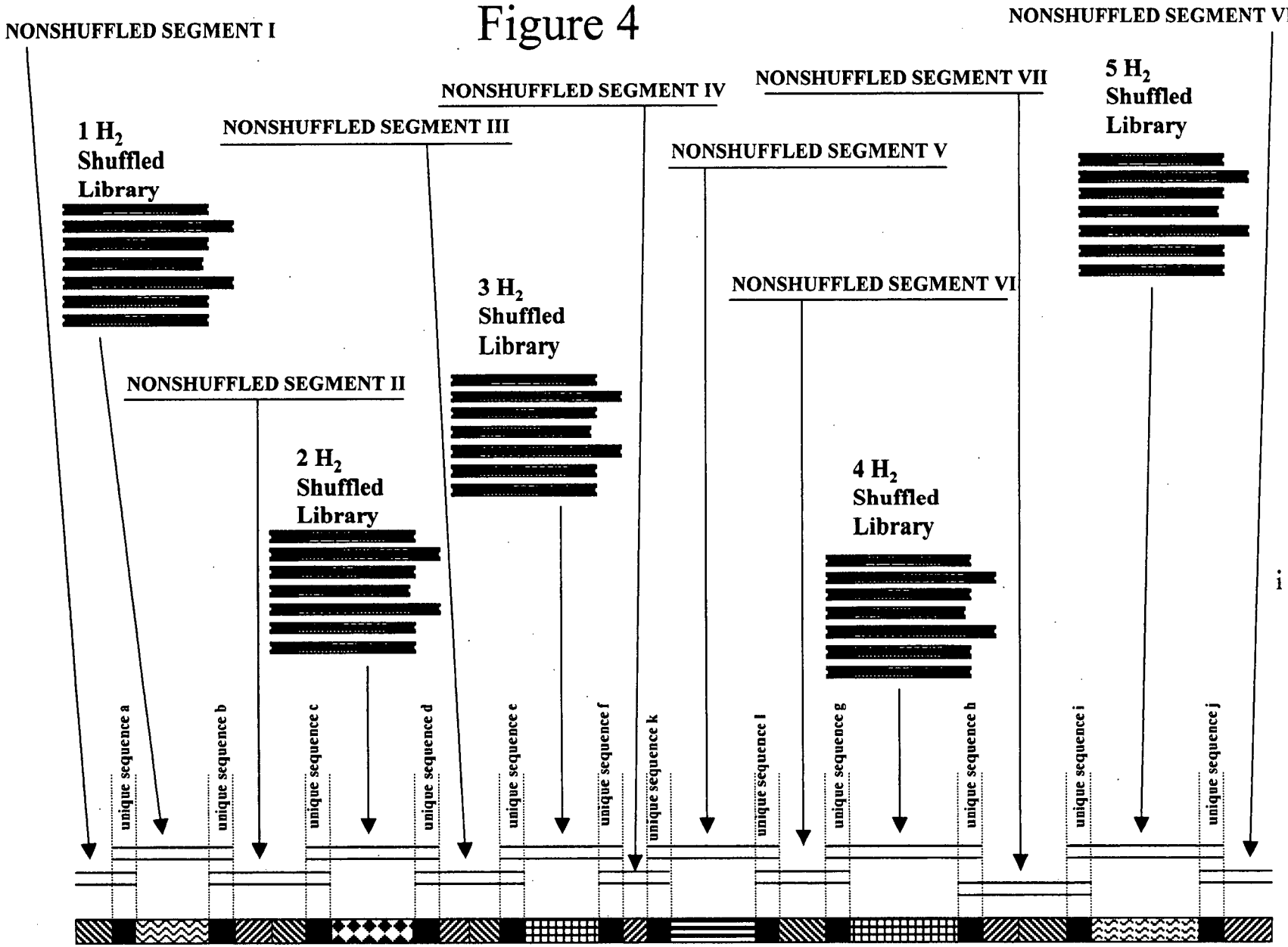


Figure 3

Extension with DNA Polymerase generates a library of test constructs with identical sequence in all regions except coding regions of shuffled versions of differentially regulated gene. The light-inducible promoter drives expression of the shuffled differentially regulated gene and the selectable or screenable marker. alternatively the selectable or screenable marker is driven by a constitutive promoter.

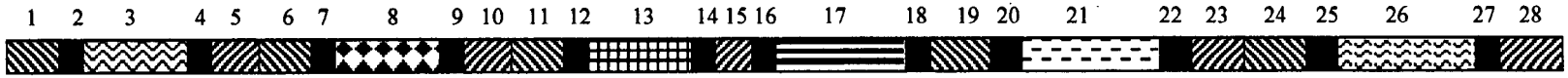
Figure 4



1-5 H_2 test construct map

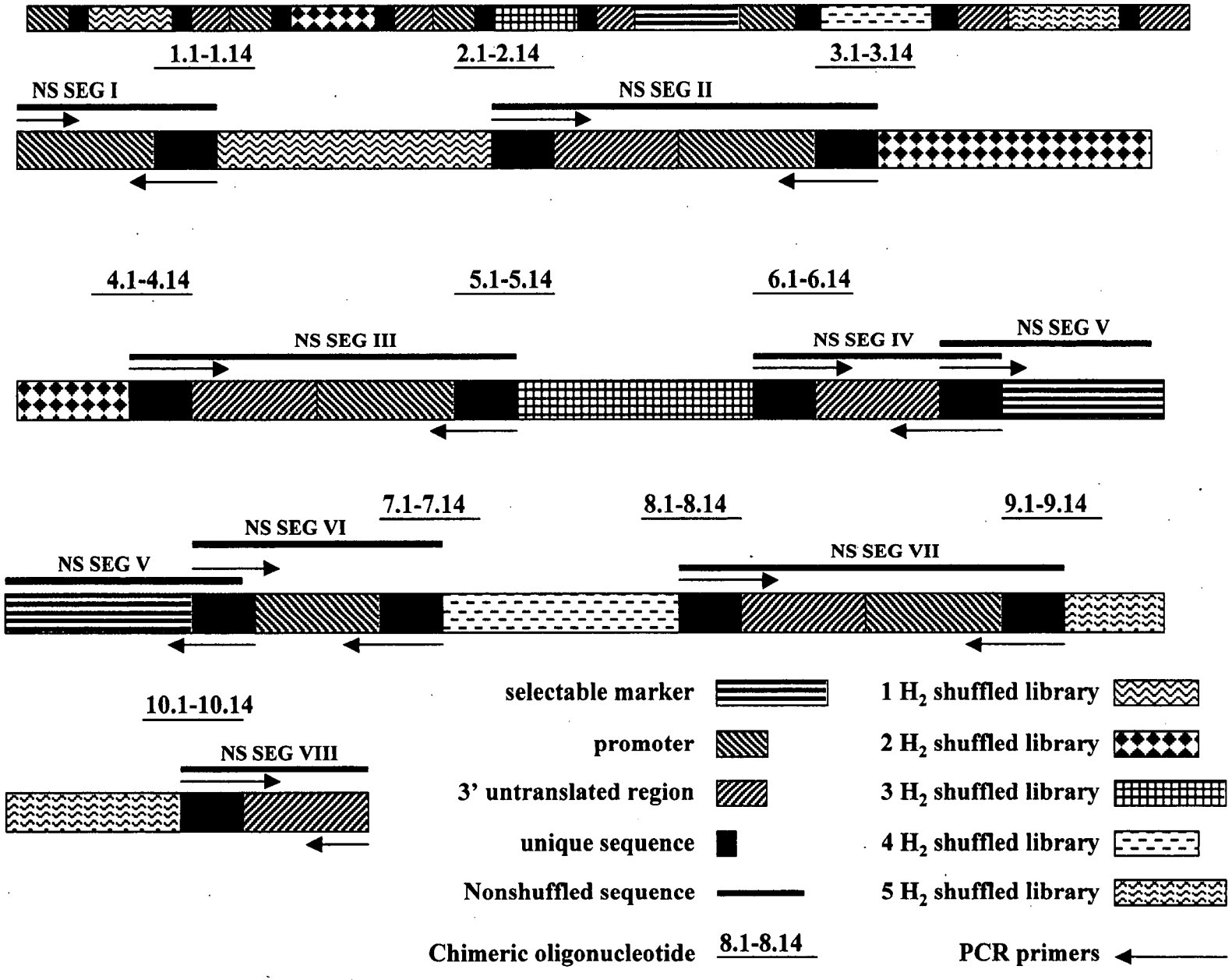
Figure 5

1-5 H_2 test constructs



1: light-sensitive promoter fragment from lhcb1 gene (SEQ ID NO 1)
2: unique sequence a (SEQ ID NO 5)
3: shuffled 1 H_2 gene library
4: unique sequence b (SEQ ID NO 6)
5: 3' untranslated region from RBCS2 gene (SEQ ID NO 3)
6: light-sensitive promoter fragment from lhcb1 gene (SEQ ID NO 1)
7: unique sequence c (SEQ ID NO 7)
8: shuffled 2 H_2 gene library
9: unique sequence d (SEQ ID NO 8)
10: 3' untranslated region from RBCS2 gene (SEQ ID NO 3)
11: light-sensitive promoter fragment from lhcb1 gene (SEQ ID NO 1)
12: unique sequence e (SEQ ID NO 9)
13: shuffled 3 H_2 gene library
14: unique sequence f (SEQ ID NO 10)
15: 3' untranslated region from RBCS2 gene (SEQ ID NO 3)
16: unique sequence k (SEQ ID NO 15)
17: ble selectable marker construct (SEQ ID NO 2)
18: unique sequence l (SEQ ID NO 16)
19: light-sensitive promoter fragment from lhcb1 gene (SEQ ID NO 1)
20: unique sequence g (SEQ ID NO 11)
21: shuffled 4 H_2 gene library
22: unique sequence h (SEQ ID NO 12)
23: 3' untranslated region from RBCS2 gene (SEQ ID NO 3)
24: light-sensitive promoter fragment from lhcb1 gene (SEQ ID NO 1)
25: unique sequence i (SEQ ID NO 13)
26: shuffled 5 H_2 gene library
27: unique sequence j (SEQ ID NO 14)
28: 3' untranslated region from RBCS2 gene (SEQ ID NO 3)

Figure 6


1.1-1.14
2.1-2.14
3.1-3.14
NS SEG I
NS SEG II
4.1-4.14
5.1-5.14
6.1-6.14
NS SEG III
NS SEG IV
NS SEG V
7.1-7.14
8.1-8.14
9.1-9.14
NS SEG VI
NS SEG V
NS SEG VII
10.1-10.14
NS SEG VIII
selectable marker
promoter
3' untranslated region
unique sequence
Nonshuffled sequence
Chimeric oligonucleotide
8.1-8.14
1 H2 shuffled library
2 H2 shuffled library
3 H2 shuffled library
4 H2 shuffled library
5 H2 shuffled library
PCR primers

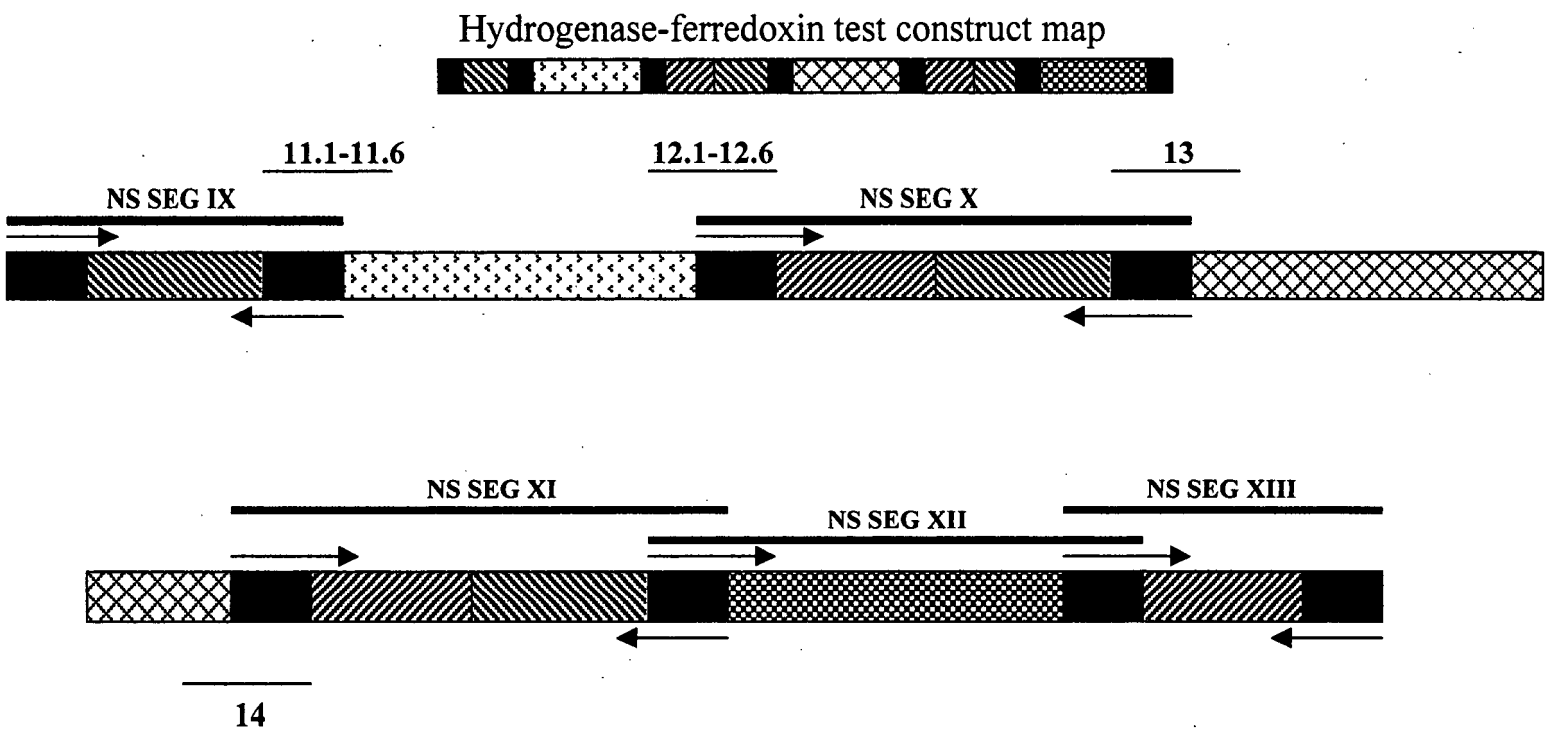


Figure 7

Shuffled hydrogenase library

Ferredoxin library

Green fluorescent protein

13.1-13.6 Chimeric oligonucleotide

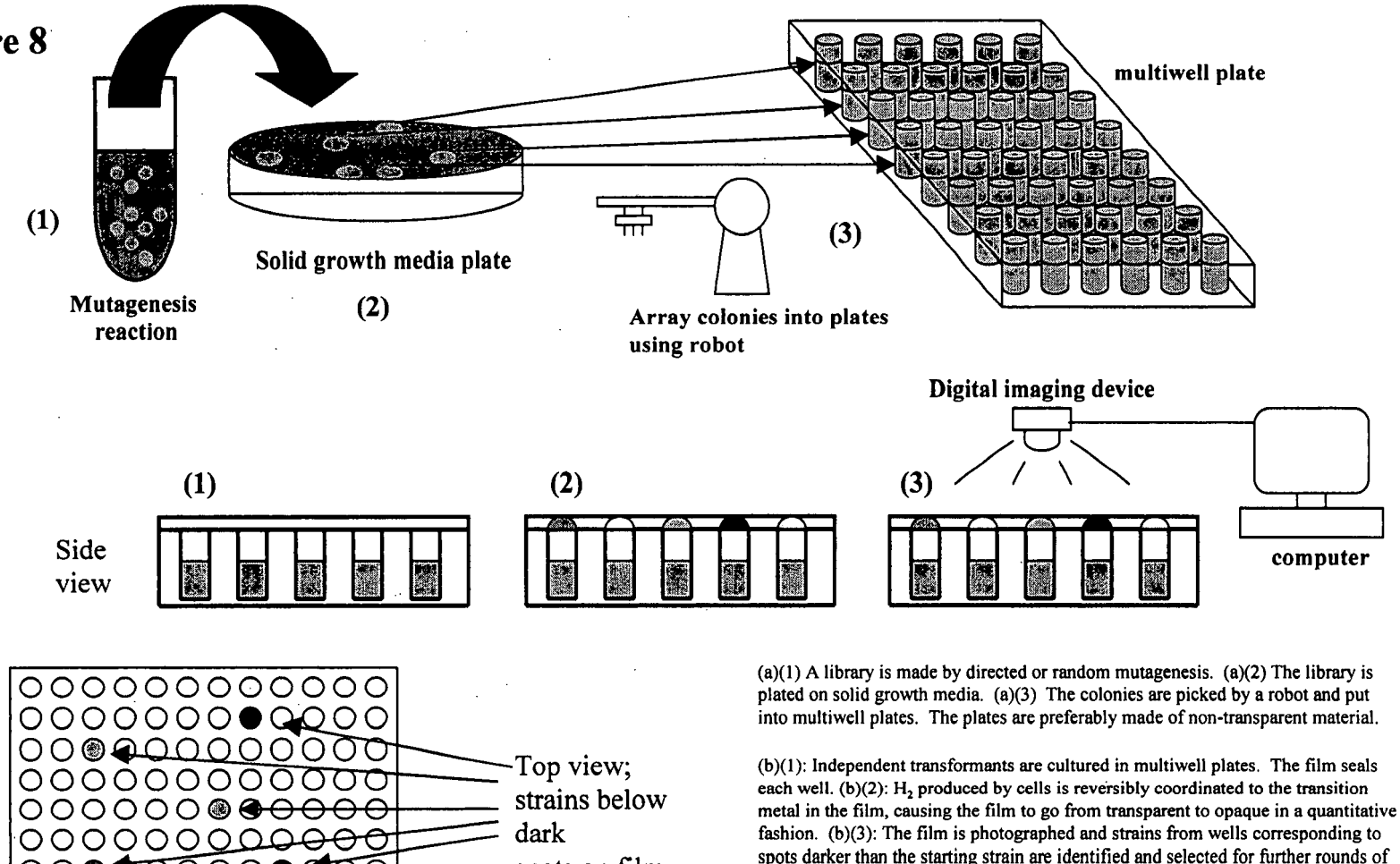
Unique sequence

3' untranslated region

promoter

PCR primer

Figure 8



(a)(1) A library is made by directed or random mutagenesis. (a)(2) The library is plated on solid growth media. (a)(3) The colonies are picked by a robot and put into multiwell plates. The plates are preferably made of non-transparent material.

(b)(1): Independent transformants are cultured in multiwell plates. The film seals each well. (b)(2): H_2 produced by cells is reversibly coordinated to the transition metal in the film, causing the film to go from transparent to opaque in a quantitative fashion. (b)(3): The film is photographed and strains from wells corresponding to spots darker than the starting strain are identified and selected for further rounds of mating and mutagenesis.

(c) Strains below dark spots on film are selected for further rounds of mating and mutagenesis.

Figure 9

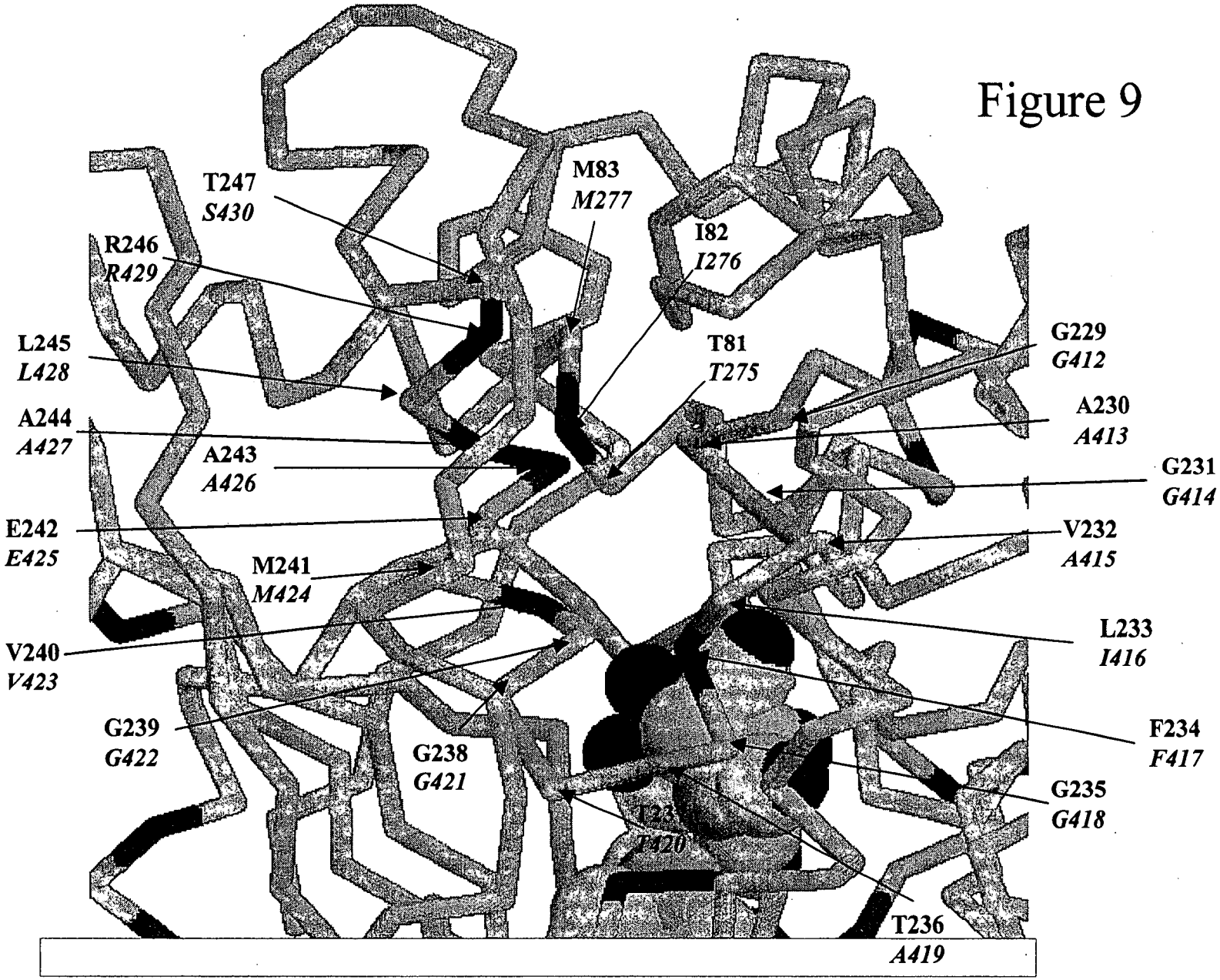

T247
S430
M83
M277
R246
R429
I82
I276
L245
L428
T81
T275
G229
G412
A244
A427
A243
A426
A230
A413
G231
G414
E242
E425
M241
M424
V232
A415
L233
I416
V240
V423
G239
G422
G238
G421
F234
F417
G235
G418
T236
A419

Figure 10

C. Reinhardtii Codon Usage Table: most preferred codons are shown underlined and in bold-face type

TTT 4.8 Phe	TCT 4.7 Ser	TAT 2.6 Tyr	TGT 1.4 Cys
TTC 29.0 Phe	TCC 16.8 Ser	**TAC 23.8 Tyr**	**TGC 12.8 Cys**
TTA 0.7 Leu	TCA 3.0 Ser	**TAA 1.2 STOP**	TGA 0.5 STOP
TTG 3.7 Leu	TCG 16.2 Ser	TAG 0.4 STOP	**TGG 13.5 Trp**
CTT 4.5 Leu	CCT 7.1 Pro	CAT 2.3 His	CGT 5.2 Arg
CTC 12.4 Leu	**CCC 29.9 Pro**	**CAC 17.5 His**	**CGC 35.3 Arg**
CTA 2.4 Leu	CCA 4.4 Pro	CAA 4.2 Gln	CGA 1.8 Arg
CTG 65.4 Leu	CCG 18.6 Pro	**CAG 36.2 Gln**	CGG 9.7 Arg
ATT 9.0 Ile	ACT 5.6 Thr	AAT 2.8 Asn	AGT 2.4 Ser
ATC 28.0 Ile	**ACC 29.9 Thr**	**AAC 29.9 Asn**	**AGC 20.8 Ser**
ATA 0.9 Ile	ACA 3.7 Thr	AAA 2.2 Lys	AGA 0.6 Arg
ATG 26.8 Met	ACG 14.7 Thr	**AAG 46.6 Lys**	AGG 2.5 Arg
GTT 5.3 Val	GCT 17.6 Ala	GAT 7.0 Asp	GGT 10.3 Gly
GTC 16.3 Val	**GCC 55.2 Ala**	**GAC 41.6 Asp**	**GGC 63.2 Gly**
GTA 2.0 Val	GCA 9.6 Ala	GAA 2.5 Glu	GGA 4.9 Gly
GTG 45.6 Val	GCG 38.6 Ala	**GAG 53.4 Glu**	GGG 8.3 Gly

Figure 11

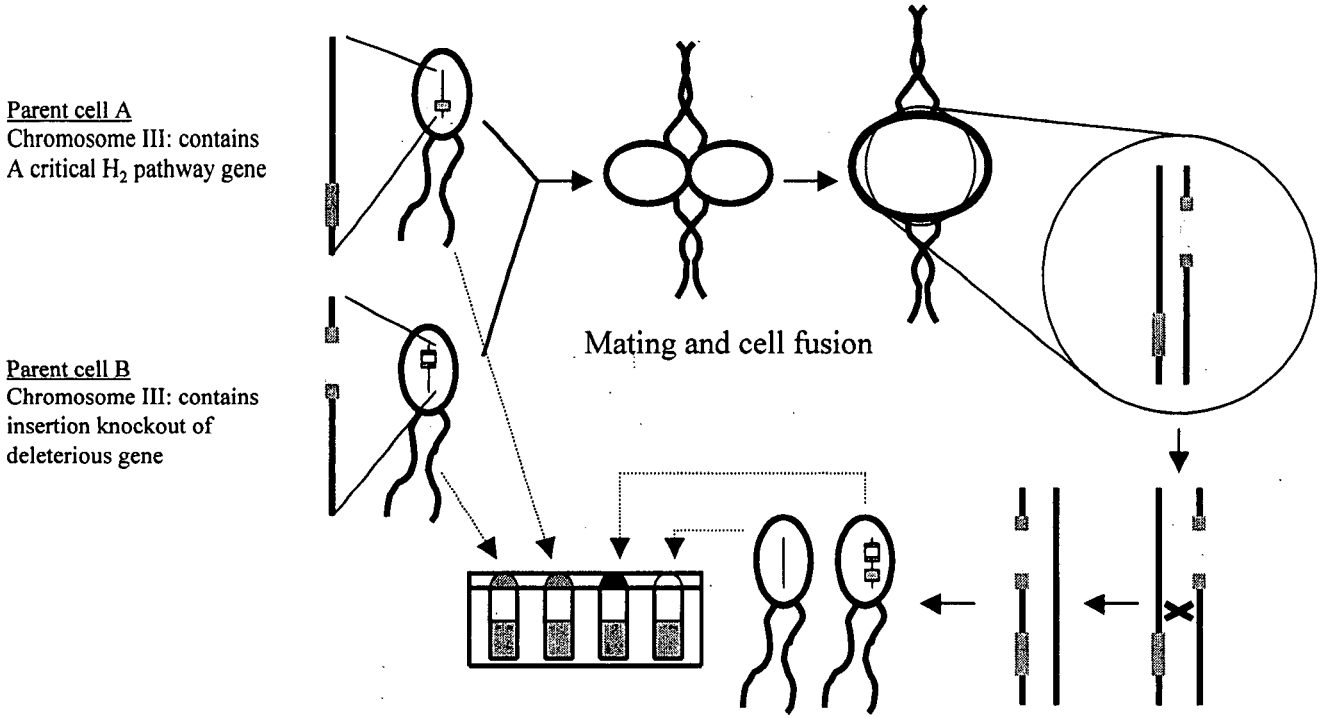

Parent cell A
Chromosome III: contains
A critical H_2 pathway gene
Parent cell B
Chromosome III: contains
insertion knockout of
deleterious gene
Mating and cell fusion

Figure 12

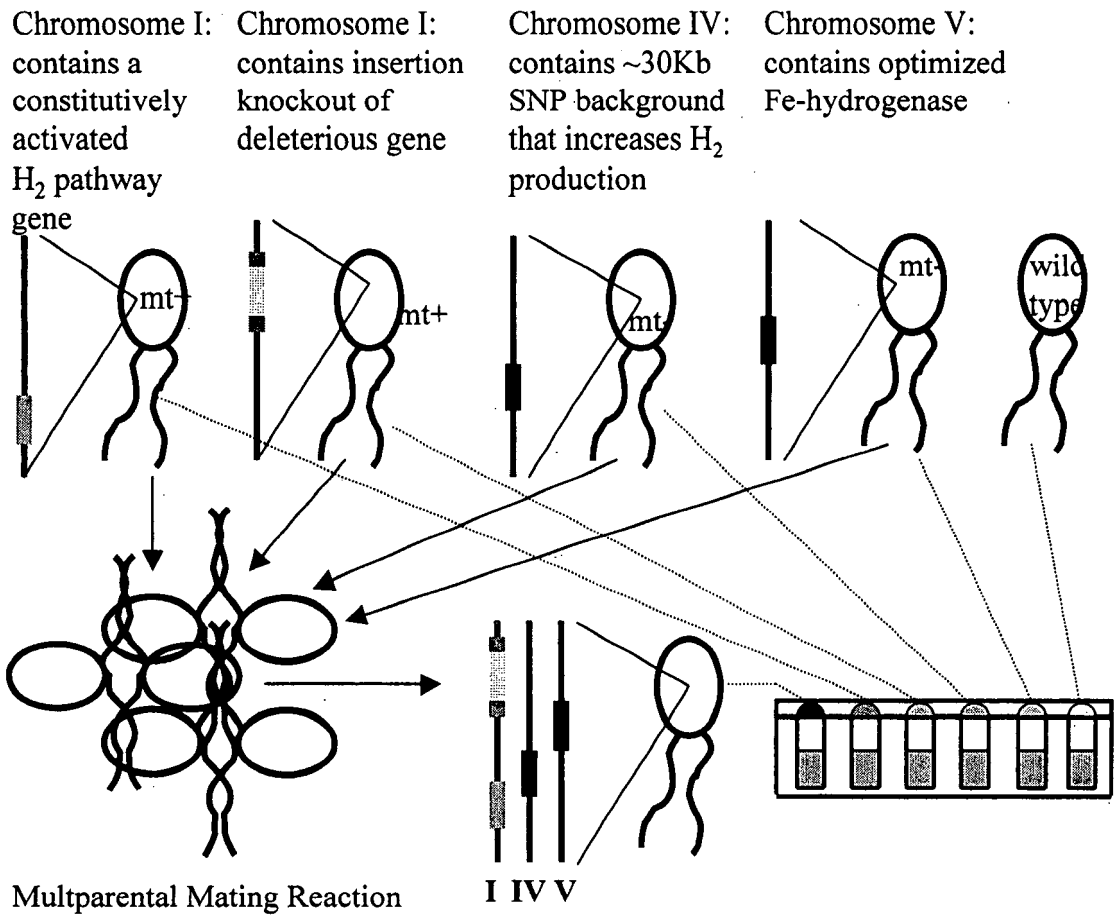

Chromosome I: contains a constitutively activated H_2 pathway gene
Chromosome I: contains insertion knockout of deleterious gene
Chromosome IV: contains ~30Kb SNP background that increases H_2 production
Chromosome V: contains optimized Fe-hydrogenase
mt-
mt+
mt-
mt-
wild type
Multparental Mating Reaction
I IV V

Figure 13

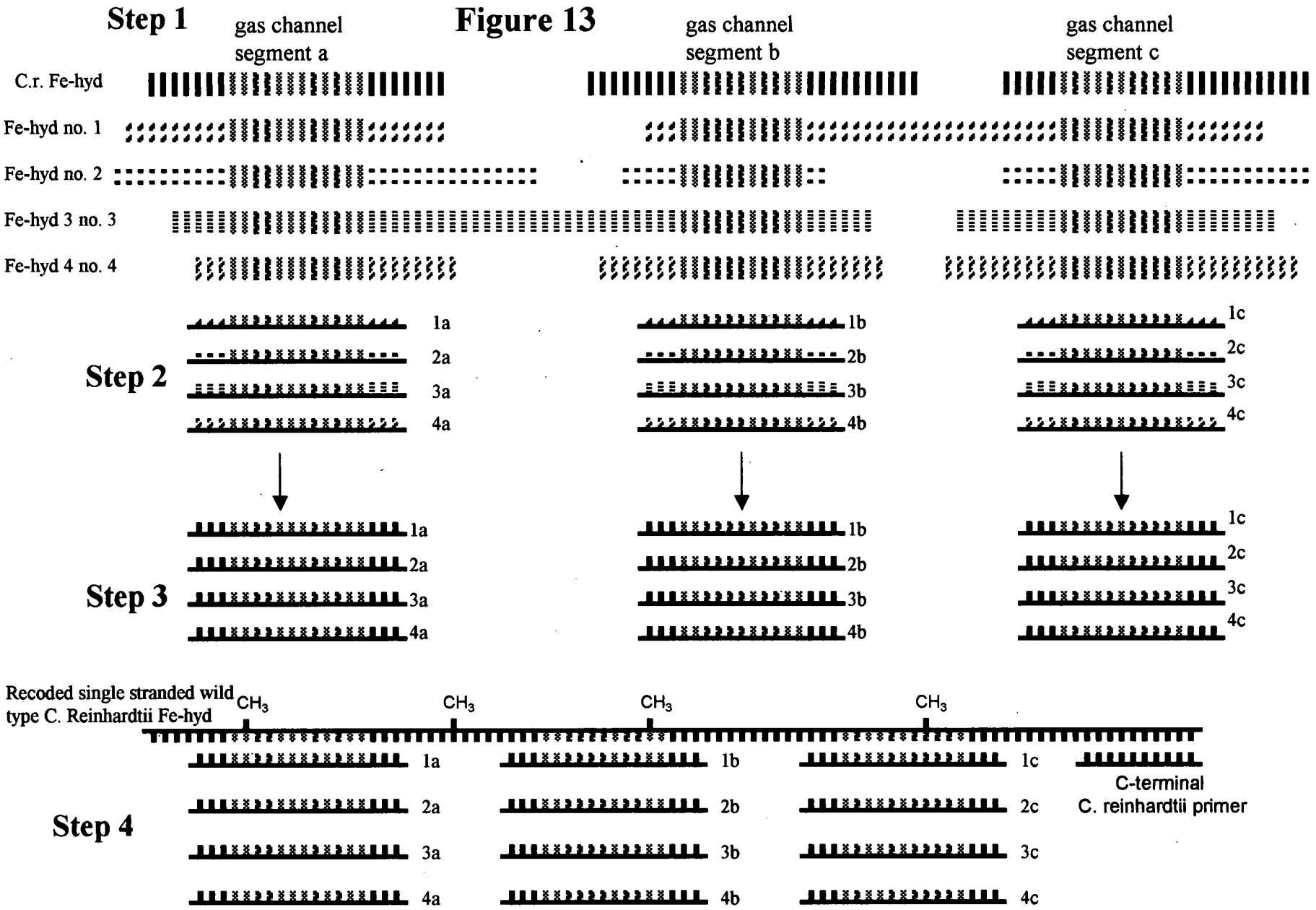

Step 1
gas channel segment a
gas channel segment b
gas channel segment c
C.r. Fe-hyd
Fe-hyd no. 1
Fe-hyd no. 2
Fe-hyd 3 no. 3
Fe-hyd 4 no. 4
Step 2
1a
2a
3a
4a
1b
2b
3b
4b
1c
2c
3c
4c
Step 3
Recoded single stranded wild type C. Reinhardtii Fe-hyd
CH3
CH3
CH3
CH3
Step 4
C-terminal
C. reinhardtii primer

Figure 14

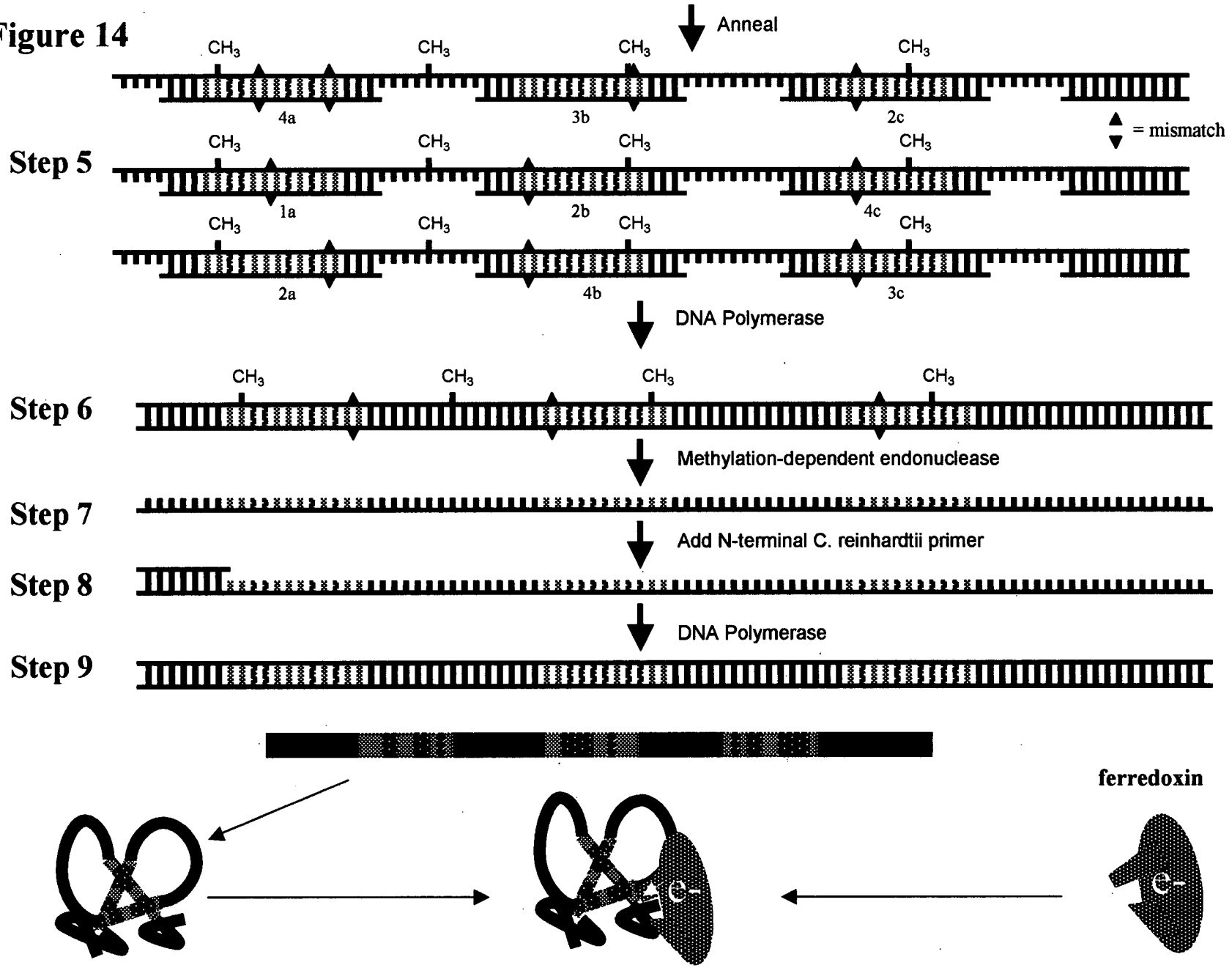

Anneal
CH3
4a
3b
2c
= mismatch
Step 5
1a
2b
4c
2a
4b
3c
DNA Polymerase
Step 6
Methylation-dependent endonuclease
Step 7
Add N-terminal C. reinhardtii primer
Step 8
DNA Polymerase
Step 9
ferredoxin
C-
C-

Figure 15

Residues within 3 amino acids of Fe-hyd most conserved residues (such as $X^1X^2X^3$ of $FX^1X^2X^3GGVMEAA$)

Most highly conserved Fe-hyd residues (such as GGVMEAA)

Framework residues of C. reinhardtii Fe-hydrogenase that are nonconserved in the Fe-hyd gene family

Framework residues of non-C. reinhardtii Fe-hydrogenase no. 1 that are nonconserved in the Fe-hyd gene family

Framework residues of non-C. reinhardtii Fe-hydrogenase no. 2 that are nonconserved in the Fe-hyd gene family

Framework residues of non-C. reinhardtii Fe-hydrogenase no. 3 that are nonconserved in the Fe-hyd gene family

Framework residues of non-C. reinhardtii Fe-hydrogenase no. 4 that are nonconserved in the Fe-hyd gene family

Figure 16

GGVMEAA Aligned Segments from SEQ ID NOs: 1-122: (corresponding to SEQ ID Nos: 124-141)

1. GAGVIFGATGGVMEAALRT
2. GGGAIFCATGGVMEAAVRS
3. GGATIFGVTGGVMEAALRF
4. GAGAIFGATGGVMEAALRS
5. GAGAIFGATGGVMEAAIRS
6. GAAVIFGVTGGVMEAALRT
7. GAGQIFAATGGVMEAASRT
8. GGGVLFGTTGGVMEAALRT
9. GAAVIFGTTGGVMEAALRT
10. GAAPIFGVTGGVIEAALRT
11. GAGVIFGTTGGVMEAALRS
12. GAGVIFGATGGVMEAAIRT
13. SAGNLFGVTGGVMEAAIRT
14. GAGAIFGATGGVMEAALRT
15. GAGVLFGTTGGVMEAALRT
16. GAAALFGVTGGVMEAALRT
17. GAGVLFGTTGGVMEAAVRT
18. GAGTIFGTTGGVMEAALRT

TIMEE Aligned Segments from SEQ ID NOs: 1-122: (corresponding to SEQ ID Nos: 142-147)

1. TIMEE
2. TIVEE
3. TIWEE
4. TICEE
5. VIMEE
6. TARLE

Figure 17

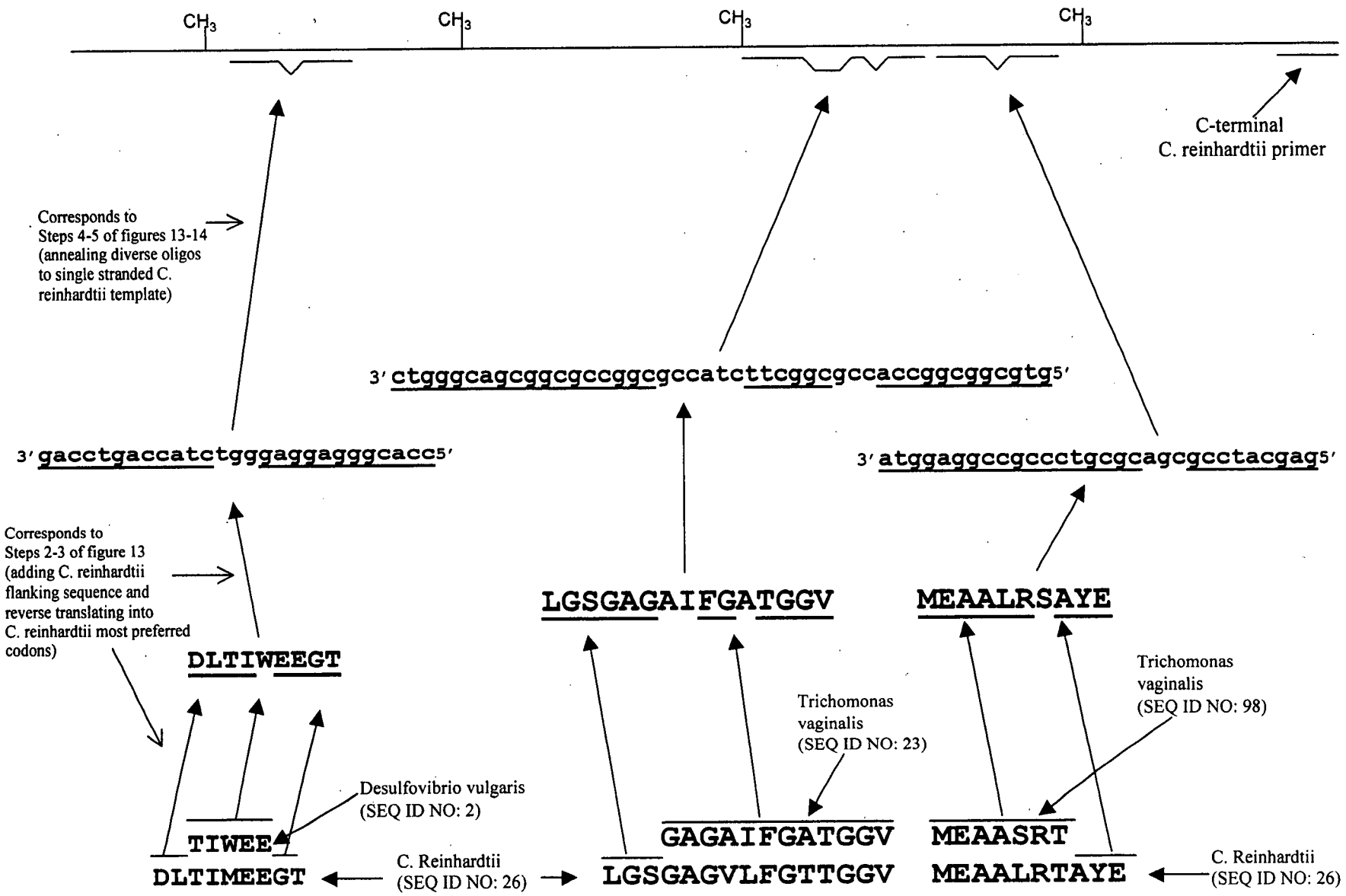
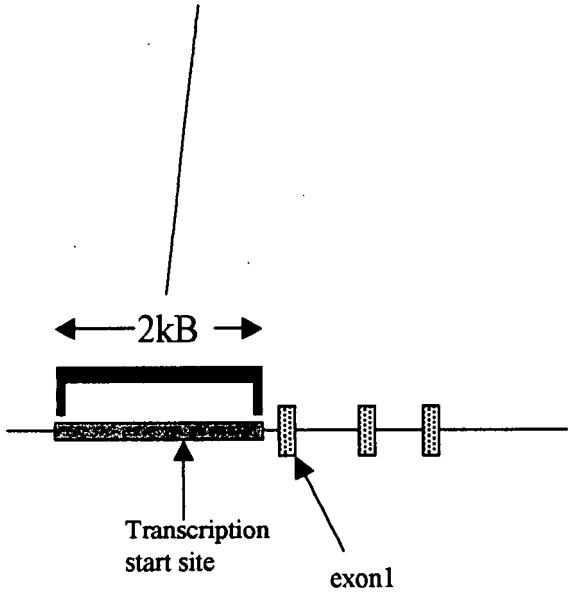
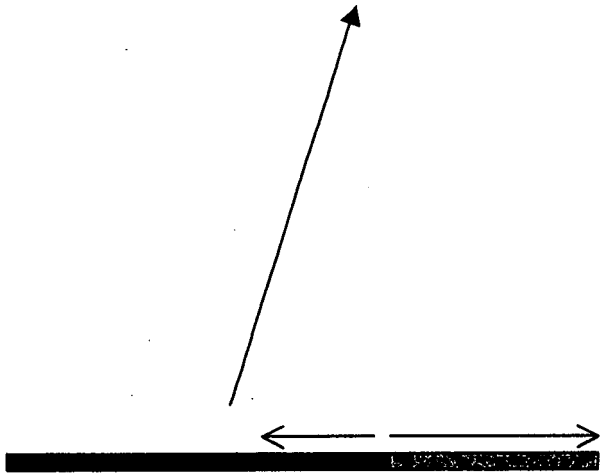

CH3
CH3
CH3
CH3
C-terminal
C. reinhardtii primer
Corresponds to
Steps 4-5 of figures 13-14
(annealing diverse oligos
to single stranded C.
reinhardtii template)
3' ctgggcagcggcgccggcgccatcttcggcgccaccggcggcgtg 5'
3' gacctgaccatctgggaggagggcacc 5'
3' atggaggccgccctgcgcagcgcctacgag 5'
Corresponds to
Steps 2-3 of figure 13
(adding C. reinhardtii
flanking sequence and
reverse translating into
C. reinhardtii most preferred
codons)
LGSGAGAIFGATGGV
MEAALRSAYE
DLTIWEEGT
Trichomonas
vaginalis
(SEQ ID NO: 23)
Trichomonas
vaginalis
(SEQ ID NO: 98)
Desulfovibrio vulgaris
(SEQ ID NO: 2)
TIWEE
GAGAIFGATGGV
MEAASRT
DLTIMEEGT
C. Reinhardtii
(SEQ ID NO: 26)
LGSGAGVLFGTTGGV
MEAALRTAYE
C. Reinhardtii
(SEQ ID NO: 26)

Figure 18

Amplify promoters from genes in nuclear, chloroplast, and mitochondrial genomes



Make insertion library with diverse promoters and screen or select for desired phenotype that arises due to altered transcriptional regulation of metabolic pathway.



Selectable marker

Connect to selectable marker through chimeric oligonucleotides and DNA Polymerase extension (marker Gene is transcribed in opposite direction)